International Journal of Bio-Technology
and Research (IJBTR)
ISSN (P): 2249-6858; ISSN (E): 2249-796X
Vol. 11 Issue 2, Dec 2021, 29–36

TRANS STELLAR
•Journal Publications •Research Consultancy

DNA FINGERPRINTING OF CMS, RESTORER, MAINTAINER LINES OF HYBRID RICE

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ABSTRACT

Rice is the main food crop for more than half of the world population. It has been estimated that the world will have to produce 60% more rice by 2030 than what is produced in 1995 (Yuan L P. 2004). Hybrid rice grows faster, yield more, and resist stress better than either parent (Yuan L P. 2012). So, hybrid rice has been proved practically for many years playing a critical role in solving the food problem by more than 20% yield advantage over improved inbred varieties (Yuan L P. 1996, 1997). Xanthomonas oryzae pv. oryzae isolates numbering 30 obtained from hybrid lines, their cytoplasmic male sterility (CMS), restorer, maintainer and inbred lines, revealed a greater amount of genetic diversity. Fifteen lineages were detected at a similarity level of 60%. The result emphasizes that these pathotypes are mismatched with the genes, xa-5, xa-13 and Xa-21 suggesting the possibility of pyramiding them for enhancing the resistance in the rice hybrids.

KEYWORDS: Hybrid Rice, Bacterial Leaf Blight & DNA Fingerprinting

Received: Sep 07, 2021; **Accepted**: Sep 27, 2021; **Published**: Nov 01, 2021; **Paper Id.**: IJBTRDEC20215

Original Article

INTRODUCTION

Rice is the main food crop for more than half of the world population. It has been estimated that the world will have to produce 60% more rice by 2030 than what was produced in 1995 (Yuan L P. 2004). Rice is a self-pollinating plant. Because of this feature, it was long assumed that developing a hybrid variety was not possible. Yuan's work reversed this assumption (Yuan L P. 1992). So, hybrid rice has been proved practically for many years playing a critical role in solving the food problem by more than 20% yield advantage over improved inbred varieties (Yuan L P. 1996, 1997). Hybrid rice is grown in an extremely wide range of climatic conditions and encounters several biotic and abiotic stresses. Bacterial leaf blight caused by *Xanthomonas oryzae* pv. *oryzae* is one of the biotic stresses and the loss measured by this pathogen is massive. This disease spreads through water and enters into the plant by leaf cuts, wounds and stomatal openings and further, it turned into a serious problem for the production of rice. There is no such chemical effective against this disease. So, growing resistant varieties is the only solution for minimizing the losses caused by this disease. It is economical and moreover an environmentally friendly method.

MATERIALS AND METHODS

Plant Materials and Culture

Leaf samples are collected from different varieties of bacterial leaf blight diseased rice plants. For this study, hybrid rice varieties and their cytoplasmic male sterility lines, restorer, maintainer and inbred lines are collected from the research plots of NRRI, Cuttack (table 1and fig. 1). Plants are grown in earthen pots and maintained in a

side a net house for natural photoperiodic conditions. The pots are regularly watered for the growth and development of the plants. Always maintain water level above the soil surface on the pots. Insecticide and pesticides are not used during the course of the study. At regular intervals, weeding is done in the pots, whenever needed. This study is repeated four times to get better results.

Table: 1 No. of Isolates Obtained from the Different Diseased Bacterial Blight Infected Plants

S. No.	Name of the Variety	No. of Isolates
1	Hybrid	17
2	Cytoplasmic male sterility lines	03
3	Maintainer lines	02
4	Restorer lines	02
4	Inbred culture	06

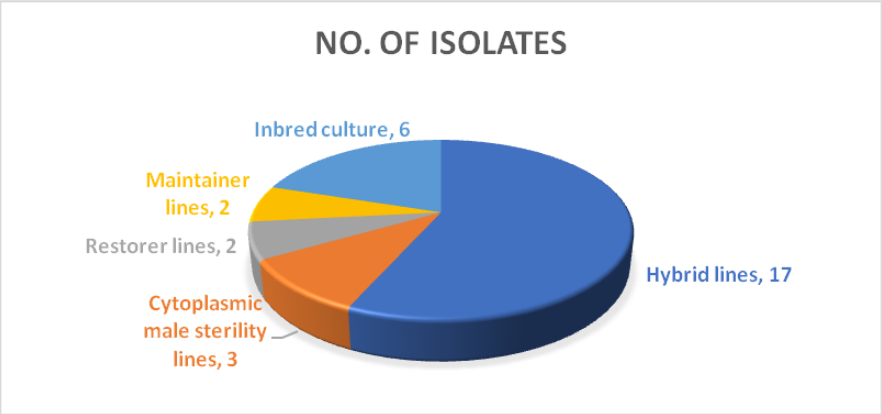


Figure 1: Distribution of Isolates from Diseased Bacterial Blight rice Varieties of Hybrid Rice Varieties and their Cytoplasmic Male Sterility Lines, Restorer, Maintainer and Inbred Lines.

ISOLATION AND MAINTENANCE OF THE BACTERIUM

Composition of modified Wakimoto medium: 20 g of sucrose, 5 g of peptone, 0.5 g of Calcium Nitrate ($Ca(NO_3)_2 \cdot 4H_2O$), 1.82 g of disodium hydrogen phosphate ($Na_2HPO_4 \cdot 7H_2O$), 0.05 g of ferrous sulphate ($FeSO_4 \cdot 7H_2O$), 18 g of agar per liter and pH 6.8-7.2 before sterilization.

Composition of medium-term storage: 100.0 gm of Skimmed milk powder, 5.0 gm of Mono-sodium glutamate per liter and 6.5 pH before sterilization (Nelson et al., 1994).

Rice leaf having young developing lesions of bacterial leaf blight pathogen (*X. oryzae* pv. *Oryzae*) symptoms of 5 to 10 cm length is collected from the field. The leaf lesions are surface sterilized by using 100 % ethyl alcohol for 30 sec. (Yoshida. 1981). The surface of sterilized leaf is sliced into small bits and placed in pre-sterilized eppendorf tubes containing 1ml of distilled water. This enables bacterial cells to ooze out in sterile water. An aliquot of 10 to 20 µl of the bacterial suspension is streak plated on the surface of the modified Wakimoto medium in Petri dishes and are placed in an incubator. All the above operations were carried out under sterile aseptic conditions. Bacterial leaf blight cultures are stored in sterile skimmed milk medium for further use.

CULTURING AND ISOLATION OF DNA FROM THE BACTERIUM

Composition of nutrient broth: 10 g of peptone, 5gm of sodium chloride (NaCl) and 3 gm of beef extract, pH: 7.2-7.5 at 28±2 °C,

DNA isolation is carried out by taking 1-2 loopful of 2/3-day old bacterial culture inoculated in an aliquot of 6 ml of nutrient broth under aseptic conditions for overnight on a rotary shaker of 100-120 rpm. An amount of 2 ml overnight broth culture is transferred to 2 ml eppendorf tube and centrifuged at 10,000 g for 2 min. The supernatant is discarded. This step is repeated more times for obtaining a good amount of pellet. The pellet is vortexed to suspend the bacterial cells (George *et al*., 1997).

EXTRACTION OF DNA FROM THE BACTERIUM

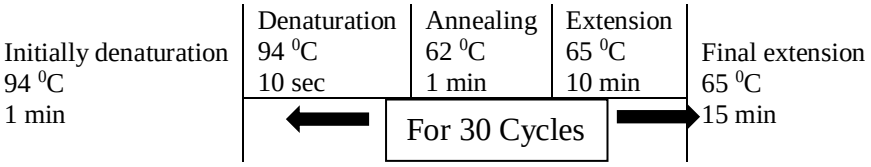
Composition of DNA extraction buffer: 100 mM Tris-HCl, pH 8.0; 100 mM of ethylenediaminetetraacetic acid (EDTA); 250 mM NaCl; 1% sodium dodecyl sulphate (SDS); 1% polyvinylpyrrolidone (PVP), 3 M potassium acetate (CH_3COOK) and 2 M acetic acid (CH_3COOH) with pH 4.8.

DNA extraction is carried out by using the potassium acetate method. To the above pellet, an amount of 650 µl extraction buffer is added and the contents are vortexed. The cell components are incubated at 65 $^{\circ}C$ for 30 min in a dry bath. The tubes are flipped rarely for uniformity of suspension. To this mixture, 100 µl of CH_3COOK is added and the contents are mixed thoroughly by mild inversions. Further, the tubes are incubated in the freezer (-20 $^{\circ}C$) for 10 min and centrifuged at 10,000 g for 15-20 min. 650 µl supernatant is carefully transferred into new eppendorf tubes. DNA is precipitated by the addition of 500 µl of ice-cool iso-propanol by slow upturns. During this step thread-like DNA strands are visible. DNA is pelleted by centrifuging at 10,000 g for 5 min. Supernatant is removed and washed with 500 µl of 70 % ethanol. The DNA pellet is air dried completely at room temperature for 4 hr. DNA is dissolved in 100 µl of ethyl alcohol and stored in the freezer (-20 $^{\circ}C$) for further use.

PCR REAGENTS

Composition of PCR reaction mixture: Nuclease free water, assay buffer (100 mM Tris, pH 8.3; 500 mM KCl; 1.5 mM $MgCl_2$ and 0.01% gelatin w/v), 185 µM dNTPs mix (dATP, dGTP, dCTP and dTTP), 0.5 µM each of two opposing primers *JEL*1(5' CTC AGG TCA GGT CGC C 3') and *JEL* 2, (5' GCT CTA CAA TCG TCC GC 3'), 20 ng of genomic DNA, 2.5 units of *Taq* polymerase and 10 % dimethyl sulfoxide (v/v) (George *et al*., 1995).

PCR is carried out by adding reagents in the vials to promote the amplification of long DNA fragments. Mix the reagents gently in a vial and add mineral oil to prevent evaporation. PCR reaction mixture is subjected to 30 cycles by using a DNA thermal cycler (Cheng *et al*., 1994). The following reaction conditions are:



VISUALIZATION OF PCR PRODUCTS

Composition of 5X TBE buffer: 89 mM Tris, pH 7.8; 89 mM boric acid; and 2 mM EDTA

To amplify DNA fragments, 10 µl of polymerase chain reaction product is mixed with 5 µl of 5 X gel loading buffer. A gel is prepared by mixing 0.5 % agarose and 0.75 % syner-gel with 0.5 X tris-borate buffer. The PCR samples are electrophoresed for seven hours at 100 V and visualized by ethidium bromide of concentration 1µg/ml. The gel is

documented and analysed with Bio-Capt software.

STUDY OF BACTERIAL LEAF BLIGHT PATHOGEN POPULATION

The bacterial leaf blight pathogen population is studied by using Bio-1D++ software. The PCR product was found to be in between 100 bp to 7 kb. To study the DNA molecular level of bacterial leaf blight pathogen isolates, the band positions are measured on the basis of the presence or absence of the location indicated as 1or 0 by the software. Binary matrix is constructed by using similarities of isolates on Dices coefficient (Yap IV and Nelson RJ. 1996). UPGMA is used for constriction of dendrogram with the help of IS*1112* repetitive element, based on polymerase chain reaction data and is found in approximately 80 copies in the bacterial genome (Leach *et al*., 1990). Groups are created with the similarity coefficient to study their percentage homology by using software Bio-1D++. Some isolates show significant homology, which represents as a unique group.

RESULT

Molecular Analysis of Pathogen Population

Hybrid rice lines are highly susceptible to many diseases and insect pests (Reddy *et al*., 1996) and bacterial leaf blight disease is one of the devastating restrictions in neutralizing their high yield potential (Raymundo *et al*., 1990). To understand the molecular level of bacterial leaf blight pathogen isolates infecting inbred cultivars/lines are diverse from those affecting hybrid lines, their CMS, restorer, maintainer and inbred lines, a set of 30 pathogen isolates are obtained from the hybrid lines grown in the experimental fields of NRRI (Table: 2). The fingerprint data of 30 isolates revealed a greater amount of genetic diversity (Ogawa T *et al*., 1991). A set of 15 isolates fingerprint from this population are presented in Figure. 3. Although, at a 30% similarity level these isolates are grouped into two main clusters and one inconsequential cluster. 15 lineages are distinguished at a similarity level of 60 %, amongst seven lineages consisting of only a single isolate.

DISCUSSIONS

The fingerprint of 30 isolates revealed a greater amount of genetic diversity of the pathogen is detected. A set of 15 Fingerprints from this population are presented in Figure. 2. Although, at a 30% similarity level these isolates are grouped into two main clusters and one inconsequential cluster Figure. 3.

Table: 2. Bacterial blight Isolates Obtained from Cultivars of Different Parental Origin

S. No.	Isolate Name	Classification
1	RH 10	Pusa hybrid
2	CR 839	Inbred culture
3	CR 749-20-2 (1)	Inbred culture
4	CRHR 4 (2)	CRRI hybrid
5	CR 679-2	Inbred culture
6	PA 6201 (3)	Pro-Agro hybrid
7	PHB 71 (1)	PAU hybrid
8	PHB 71 (3)	PAU hybrid
9	NSD-2 (1)	NDUAT hybrid
10	CRHR 5 (1)	CRRI hybrid
11	PK 117 R	PAU hybrid
12	CRHR 1	CRRI hybrid
13	PAC 89001	ITC hybrid
14	IET 16645	Inbred culture

15	HKRH 1008	Indo-American hybrid
16	CR 2007-`1	Inbred culture
17	IR 42266	Restorer line
18	NSD-2 (2)	NDUAT hybrid
19	MPH 525	Mahyco hybrid
20	MTURH 2020	Maruteru hybrid
21	CR 1014	Inbred culture
22	DRRH 1	DRR hybrid
23	IR 53258	Restorer line
24	CRMS 29A	CMS line
25	CRMS 31A	CMS line
26	CRMS 32A	CMS line
27	IR 62829A	Maintainer line
28	IR 62829B	Maintainer line
29	CRHR 4 (1)	CRRI hybrid
30	CRHR 4 (3)	CRRI hybrid

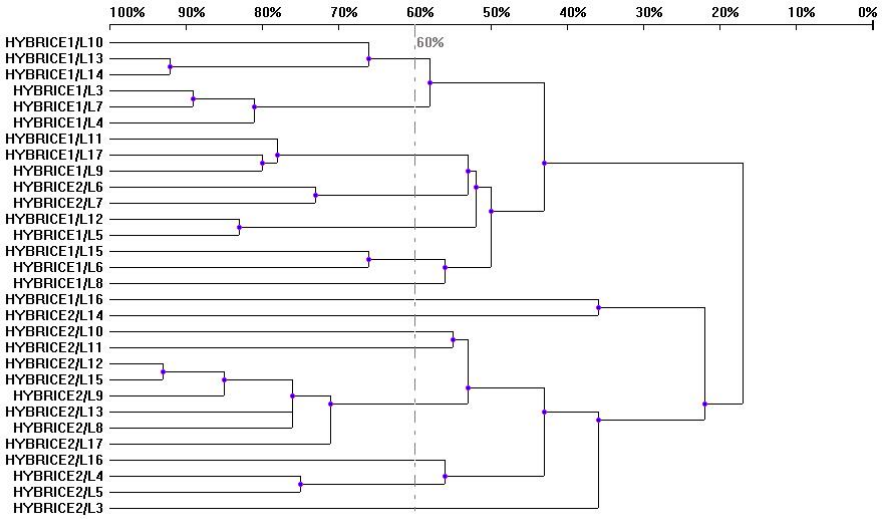


Figure 3: Dendrogram Constructed by using 30 Isolates of Bacterial Leaf Blight from Cytoplasmic Male Sterile, Restorer, Inbred and Hybrid Rice Lines. Homology Level of 60 % is Marked with Broken Lines Vertically.

CONCLUSIONS

Xanthomonas oryzae pv. *oryzae* isolates of number 30 are obtained from different rice varieties of hybrids from all over India, Cytoplasmic male sterility lines (CMS), restorer, maintainer and inbred lines, revealed a greater amount of genetic diversity. Only fifteen lineages were detected at a similarity level of 60%. No specific differences among these varieties of pathogen isolates were observed.

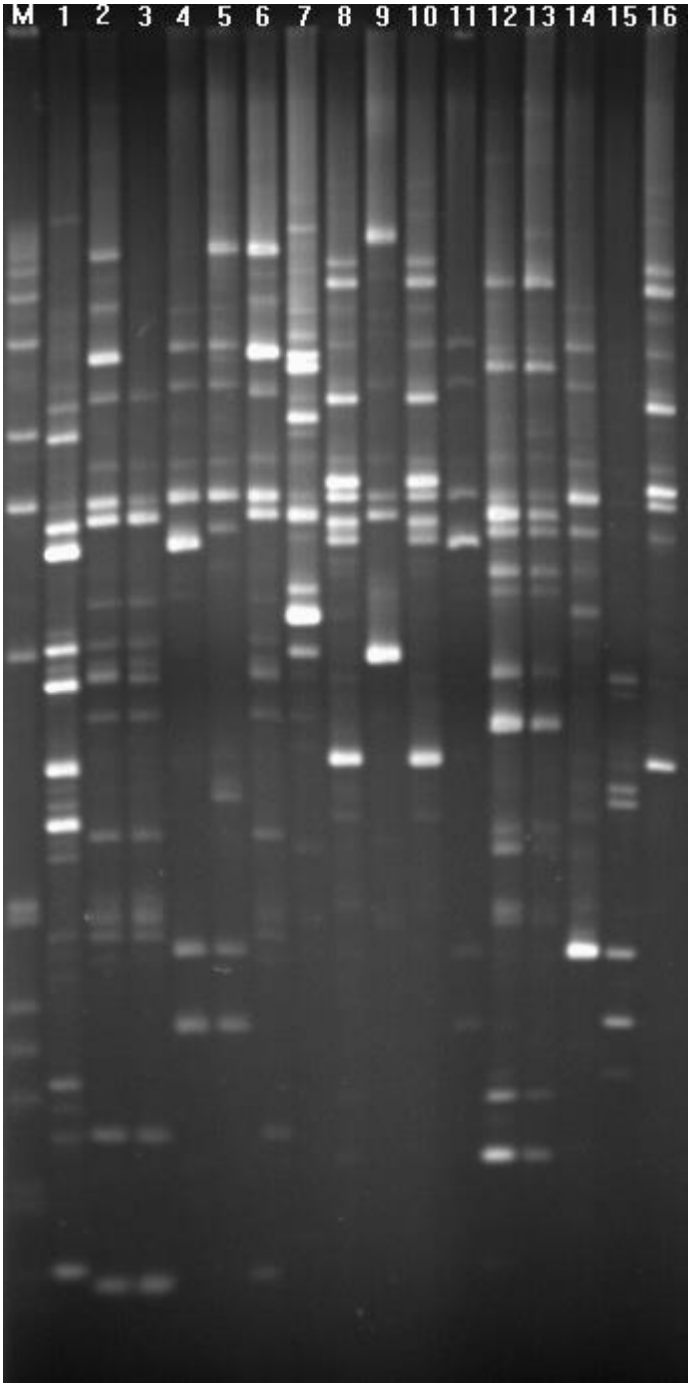


Figure 2: PCR Fingerprints of Bacterial leaf Blight Isolates Originating from Hybrid Rice, and Cytoplasmic Male Sterile Lines were Grown at the Experimental Fields of NRRI. Lane Marked M is DNA Molecular Size Marker, 1-kb Ladder; Lane 1 Contains the Fingerprint of Reference Isolate IAXO 1486. Lanes 2 to 16 Contain the Fingerprints of Isolates; 2: RH-10; 3: CR 839; 4: CR 749-20-2(1); 5: CRHR-4(2); 6: CR 679-2; 7: PA-6201(3); 8: PHB-71(1); 9: PHB-71(3); 10: NSD-2(1); 11: CRHR-5; 12: PK 117 R; 13: CRHR-1; 14: PAC 89001; 15: IET 16645 and 16: HKRH 1008.

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